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Slit-Roundabout Signaling Neutralizes Netrin-Frazzled-Mediated Attractant Cue to Specify the Lateral Positioning of Longitudinal Axon Pathways

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ABSTRACT

An extending axon growth cone is subjected to attractant and repellent cues. It is not clear how these growth cones discriminate the two opposing forces and select their projection paths. Here, we report that in the Drosophila nerve cord the growth cones of longitudinal tracts are subjected to attraction by the Netrin-Frazzled pathway. However, the midline Slit neutralizes this pathway in a Robo-dependent manner and prevents Netrin-Frazzled-mediated attraction of longitudinal tracts. Our results suggest that the loss of a neutralizing effect on the Netrin-mediated attraction is responsible for the longitudinal tracts entering the midline in *slit* mutants as opposed to a loss of repulsion as is currently believed. This effect is not via a direct inhibition of Frazzled by Robo; instead, it is at a level downstream of Frazzled. Thus, the growth cones of longitudinal tracts subjected to two opposing forces are able to block one with the other and specify their correct lateral positioning along the midline.

IN both insects and vertebrates, chemorepellents and chemoattractants provide guidance cues to axons and regulate their pathfinding along stereotypical pathways toward their synaptic targets (reviewed in Tessier-Lavigne 1994; Goodman 1996; Van Vactor and Flanagan 1999; Harris and Holt 1999; Seeger and Beattie 1999). It is currently thought that membrane-bound receptors and secreted molecules function as either attractants or repellents mediating short-range and/or long-range signaling during this process. Thus, activities of axon repellents and axon attractants regulate the formation of commissures, the axon tracts that cross the midline and connect the two sides of the CNS, and longitudinal connectives, the axon tracts that travel along the nerve cord and connect different segments along the anterior-posterior axis.

Recently, much effort has been directed toward elucidating axon repulsion mediated by the Slit (Sli)-Roundabout (Robo) signaling (Ba-Charvet *et al.* 1999; Brose *et al.* 1999; Kidd *et al.* 1999; Li *et al.* 1999; Wang *et al.* 1999; Bashaw *et al.* 2000; Rajagopalan *et al.* 2000; Simpson *et al.* 2000) and axon attraction mediated by the Netrin (Net)-Frazzled (Fra) signaling (Harris *et al.* 1996; Kolodziej *et al.* 1996; Mitchell *et al.* 1996). For instance, it has been shown that growth cones that express Robo, Robo2, and Robo3, the receptors for Sli, will not enter and cross the midline where *sli* is expressed at high levels. Thus, in *robo* mutants axons freely cross and recross the midline whereas in *sli* mutants, axons that normally do not enter the midline freely do so but do not leave the midline after entering (Kidd *et al.* 1999; Bashaw *et al.* 2000; Rajagopalan *et al.* 2000; Simpson *et al.* 2000). The major conclusion from these studies is that a repellent interaction between Sli and the three different Robo proteins prevents the longitudinal pathways in the ventral nerve cord from projecting toward the midline (Kidd *et al.* 1999; Bashaw *et al.* 2000; Rajagopalan *et al.* 2000; Simpson *et al.* 2000). According to this conclusion, a gradient of Sli emanating from the midline interacts with these three different Robo receptors in a combinatorial manner to specify lateral positions of axon tracts in the longitudinal pathways (Rajagopalan *et al.* 2000; Simpson *et al.* 2000). High levels of Sli interact with Robo to specify the medial tract; intermediate levels of Sli interact with Robo, Robo 3, and low levels of Robo2 to specify the intermediate tract; and the lowest levels of Sli interact with Robo, Robo3, and high levels of Robo2 to specify the lateral tracts.

Several findings argue against the above model. For instance, a gradient of Sli extending from the midline has never been detected. More importantly, overexpression of *sli* at the midline does not alter the lateral positioning of longitudinal tracts (Kidd *et al.* 1999; our unpublished data), as one would expect for a repellent signal that emanates from the midline. Furthermore, *robo2* mutants also exhibit a weak midline crossing of medial tracts phenotype (Rajagopalan *et al.* 2000), which is inconsistent with the proposed model since Robo2 is expressed only in the lateral tracts (Rajagopalan *et al.* 2000; Simpson *et al.* 2000). These results raise the possibility that the lateral positioning of longitudinal tracts by Sli-Robo signaling is much more complex than

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these studies suggest and involves additional pathways or mechanisms.

In Drosophila, Netrins (Net A and B) and their receptor, Fra (a member of the Deleted in Colorectal Cancer/UNC-40 family), first discovered and cloned and studied in *Caenorhabditis elegans* (Hedgecock *et al.* 1990; Ishii *et al.* 1992; Chan *et al.* 1996), mediate attraction of commissural growth cones toward the midline (Harris *et al.* 1996; Kolodziej *et al.* 1996; Mitchell *et al.* 1996; Stein and Tessier-Lavigne 2001). In *net* or *fra* mutant embryos the commissural tracts fail to cross the midline in a significant number of segments (Harris *et al.* 1996; Kolodziej *et al.* 1996; Mitchell *et al.* 1996). However, it is not known whether the Netrin-Fra-mediated attractant signaling has any role in the positioning of longitudinal tracts. Indeed, if these tracts are subjected to Net-mediated attraction, it is not clear how the longitudinal tract growth cones discriminate the two opposing forces (the Sli-Robo being the repellent one) and select their projection paths.

A previous study has shown that in stage 22 Xenopus spinal neurons binding of Sli to Robo leads to an interaction between the cytoplasmic domains of Robo and Fra, which in turn silences the Fra-Net-mediated attraction (Stein and Tessier-Lavigne 2001). We sought to determine if a similar interaction exists between Sli signaling and Net signaling, which then allows longitudinal tract growth cones to position along the nerve cord. In this article, we show that the growth cones of longitudinal tracts are subjected to attraction by the Net-Fra pathway. However, the midline Sli blocks this pathway in a Robo-dependent manner and prevents Net-Fra-mediated attraction. Thus, it appears that the loss of a neutralizing effect on Net-Fra-mediated attraction is responsible for the longitudinal tracts entering the midline in *sli* mutants as opposed to a loss of repulsion. Moreover, this effect is not via a direct inhibition of Fra by Robo as was shown in Xenopus using *in vitro* studies (Stein and Tessier-Lavigne 2001). Instead, it is at a level downstream of Fra. These results provide a novel insight into how the lateral tracts are positioned along the midline by two opposing cues.

MATERIALS AND METHODS

Stocks and genetics: To analyze *sli* function, sli^{2}, sli^{GA20} (null point mutations), $sli^{E\text{-}158}$ (a hypomorphic *P*-element-insertion allele of *sli*; *P*-element insertion in the promoter region, see Rothberg *et al.* 1990), and a *sli* deficiency were used. For *robo*, $robo^{4}$ (null allele) and a *robo* deficiency were used. For *net A* and *net B* double mutant, we used a small deficiency that eliminates the two genes and is well characterized for *net* phenotypes in previous studies (see Harris *et al.* 1996). For *fra*, the point mutants fra^{3} and fra^{GA957} and a deficiency for *fra* were used. Double mutants between *net* and *sli* or *robo* genes were constructed by standard genetics. The other stocks used were as follows: *UAS-netA, UAS-netB, UAS-fra, UAS-sli, UAS-robo, elav-GAL4* (a pan-neural driver), *sim-GAL4* (a midline driver), *ptc-GAL4, arm-GAL4, UAS-GAL4,* and UAS-$Robo^{extra}$-Fra^{intra}. Combinations between various UAS transgenes, GAL4 drivers, and mutants were constructed by standard genetics.

Ectopic expression of Sli: To express *sli* in RP2, in rows of cells right above the RP2 cell body, in rows of cells immediately below aCC, and in rows of cells above pCC, we used the *UAS-sli/UAS-GAL4* strategy with the *ptc-GAL4* and *arm-GAL4* as drivers (see supplementary Figure 1 at http://www.genetics.org/supplemental/ for the *ptc* and *arm* expression domains). *UAS-sli* was first introduced into *UAS-GAL4* background. These flies were then crossed to either *ptc-GAL4* or *arm-GAL4*. To have a continued clonal expression of Gal4, *UAS-GAL4* was also introduced into these backgrounds (*UAS-sli; UAS-GAL4; ptc-* or *arm-GAL4*). *UAS-GAL4* is activated initially by *ptc-* or *arm-GAL4* and then clonally maintained by the *UAS-GAL4* autoregulatory loop. For the pan-neural ectopic expression of *sli*, *UAS-sli; UAS-GAL4* flies were crossed to *elav-GAL4*.

Antibodies and immunostaining: Embryos were fixed and stained with the following antibodies: Fas II (1:5), BP102 (1:15), Sli (1:50), Eve (1:2000), Mab 22C10 (1:10), LacZ (1:3000), and Robo (1:50). For confocal microscopy, cy^{5} and FITC-conjugated secondary antibodies were used. For light microscopy, alkaline phosphatase or 3,3′-diamino-benzidine conjugated secondary antibodies were used. Mutant embryos were identified using blue balancers, marker phenotypes, or immunostaining (lack of positive staining). Please note that since a given stage can be as long as several hours or as short as 10 min, I have represented the age of the embryo in hours rather than stating the stage.

RESULTS

Netrin-Frazzled attractant signaling specifies the positioning of longitudinal tracts along the midline: To determine if the Net-Fra pathway plays any role in the positioning of longitudinal tracts, we examined embryos mutant for these genes. Previous results have shown that in embryos mutant for *fra* the guidance of commissural axons is severely affected (Kolodziej *et al.* 1996). As shown in Figure 1C, these tracts are stalled and the nerve cord exhibits reduced or missing commissural tracts. Similar commissural defects were also observed in embryos mutant for *netrins* (Figure 1B). Moreover, the tracts are frequently projecting toward the periphery (Figure 1B, black arrowhead). In both *net* and *fra* mutants, commissural defects were observed in every segment of a mutant embryo ($n = 30$ embryos). In addition to the commissural defects, the longitudinal connectives were also affected in *net* or *fra* mutants. These tracts were positioned farther away from the midline in these mutants ($n = 12$ embryos) compared to wild type (see below). That this is caused by a reduction in the commissural tracts thereby compromising the cohesiveness or tension between tracts is unlikely since we observed several segments that lacked entire commissural tracts but were not any different from the regions where there was only a reduction in the commissural tracts (*cf.* Figure 3F). Finally, since the commissural tracts are not completely eliminated in *net* or *fra* mutants (Figure 1, B and C), there must be additional attractant cues from the midline, in addition to Netrins, that the commissural tracts respond to in order to project toward the midline.

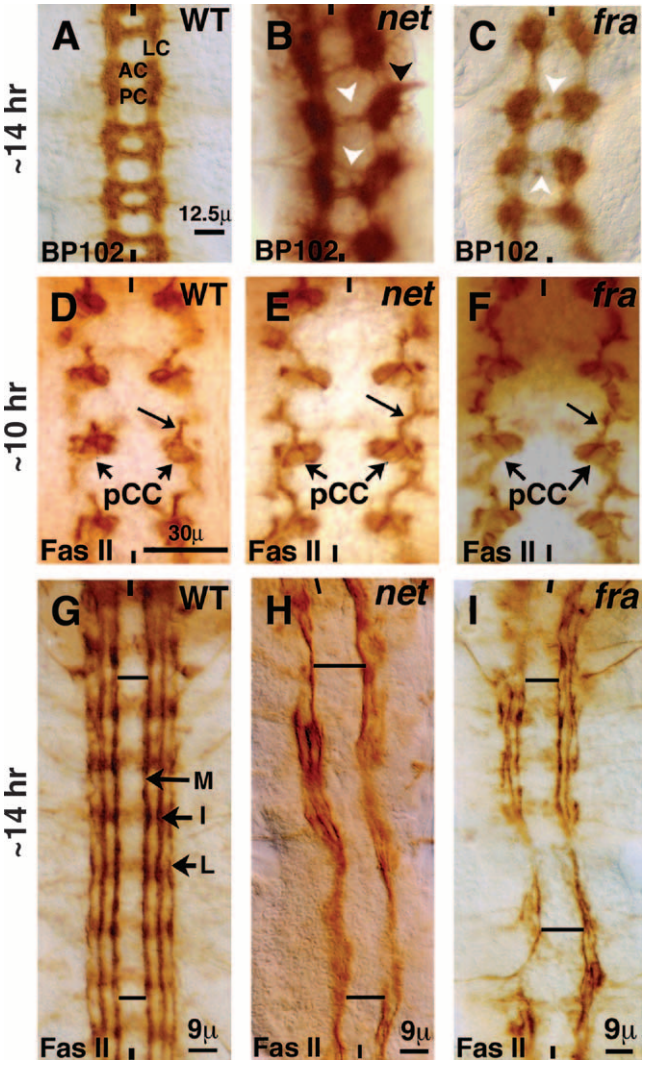

FIGURE 1.—Netrin-Frazzled signaling attracts longitudinal axon tracts toward the midline. Anterior side is up; midline is marked by vertical lines. Embryos are ~14 hr old. WT, wild type. Embryos in A–C are stained with BP102; scale bar for A–C is given in A. (A) Wild type. BP102 stains the anterior commissure (AC), the posterior commissure (PC), and the longitudinal connectives (LC). (B and C) In *net* and *fra* mutants, the connectives are farther apart and the commissures are often missing, very thin (white arrowhead), or project outward (black arrowhead). Embryos in D–I are stained for Fas II; scale bar for D–F is given in D. (D) Wild-type embryo. Fas II stains pCC (arrow) and its projection (long arrow), which pioneers the medial tract. (E and F) *net* and *fra* mutant embryos. The projections from pCC are farther apart than in wild type. (G) Wild-type embryo. Fas II stains three major longitudinal tracts, medial (M), intermediate (I), and lateral (L). (H and I) *net* and *fra* mutants. These Fas II-positive pathways are farther away from the midline and are often disorganized or missing in regions along the tract (see also Table 1).

Previous work in *C. elegans* showed that Net determines the placement of longitudinal axons with respect to the midline (REN *et al.* 1999). However, it was not known if the Net-Fra signaling also exerts an attraction on the well-studied fasciclin II (Fas II)-positive longitudinal tracts in the Drosophila embryo. We sought to examine this by staining *net* and *fra* embryos with anti-Fas II (mAb1D4). As shown in Figure 1G, in wild type Fas II stains three major longitudinal pathways: the medial pathway (closest to the midline and formed first), the lateral pathway (farthest from the midline), and the intermediate pathway (between the medial and the lateral). One of the pioneering axons for the medial tract is from the interneuron pCC. Fas II stains both pCC and its axon projection at an earlier stage of development (Figure 1D). In wild type the distance between medial tracts is progressively reduced from ~30 μm in 10-hr-old embryos to ~9 μm in 14-hr-old embryos (Table 1). When we examined 10-hr-old *net* or *fra* mutant embryos, we found that, compared to those in wild type, the tracts from pCC neurons were projected farther away from the midline (Figure 1, E and F), positioned ~40 μm and ~37 μm, respectively (Table 1); however, the difference in the position of the pCC neuron in *net* and *fra* mutants and in wild type was minimal. When we examined ~14-hr-old embryos, we found that in *net* and *fra* mutants the medial tracts were positioned ~20 μm and ~18 μm apart, respectively, while in wild type the medial tracts were ~9 μm apart (Figure 1, H and I; Table 1). These results suggest that Net, which is expressed in the midline, and Fra, which is present in the growth cones, provide an attractant signaling cue for the lateral positioning of medial tracts on either side of the midline.

The intermediate and lateral tracts in *net* or *fra* mutants did not appear to be farther away from the midline than those in wild type. However, these tracts were broken and often collapsed on each other. Since Netrins also promote axon growth, this intermittent breaking of the two lateral tracts (but interestingly not the medial tract) might be due to an effect of loss of Net activity on axon growth. Moreover, the nerve cord in the mutants was much narrower compared to that in wild type. It may be that some neurons from the lateral neuroblasts are missing in *net* or *fra* mutants, resulting in a much narrower nerve cord. This can cause broken nerve tracts and might also correct the abnormal lateral positioning of the longitudinal tracts to some extent in these mutant embryos.

We further tested the possibility that the Net-Fra signaling system influences the lateral positioning of longitudinal tracts along the midline. If Net attracts the longitudinal tracts toward the midline, an overexpression of Net in the midline should attract longitudinal tracts toward the midline and either make these tracts collapse at the midline or, at the minimum, cause a shift in the lateral positioning of longitudinal tracts closer to the midline. Indeed, when *net* was overexpressed in the midline from *UAS-net* (*net A* or *net B*) with sim-GAL4 driver in wild-type background (each in single copy), it resulted in the collapse of the longitudinal tracts at the midline (Figure 2, C and D; two copies each causes

TABLE 1

The distance between the two medial tracts across the midline at three different embryonic developmental points in wild-type and mutant backgrounds

Age (hr)	Wild type (μm)	*net* (μm)	*fra* (μm)	*net;sli* (μm)	*net; robo* (μm)
10	30 ± 1	40 ± 3	37 ± 3	41 ± 3	39 ± 3
11.5	19 ± 0.5	31 ± 3	28 ± 2	29 ± 3	29 ± 2
14	9 ± 0.5	20 ± 2	18 ± 1	21 ± 2	21 ± 3

The embryos were stained with Fas II antibody. The values for each genotype and each time point are averages taken from 12 different embryos and the distance was measured from each embryo in at least two different points along the nerve cord. Note that the distance between the two medial tracts across the midline is decreasing with embryonic development.

a stronger collapsing of longitudinal tracts, data not shown). This phenotype was observed in ~40% of the hemi-segments ($n = 336$ hemi-segments). Furthermore, the lateral positioning of the tracts in places where they have not collapsed at the midline is shifted more toward the midline (Figure 2D). Furthermore, we also overexpressed *net* in the midline in a *sli* hypomorphic mutation, sli^{E158}. This is a *P*-element-insertion allele (Kidd *et al.* 1999) and the longitudinal tracts are not as severely affected (Figure 2E) as in *sli* null embryos (Figure 3A). Overexpression of *net* at the midline in this *sli* hypomorphic background (*UAS-net* and sim-GAL4, each in single copy) caused a significant enhancement of the phenotype, and the longitudinal tracts of these embryos appeared similar to strong loss-of-function *sli* mutant embryos (Figure 2F). In summary, these results argue that Net signaling plays an attractant role in the lateral positioning of longitudinal tracts, in particular the medial tract along the midline.

***netrin/frazzled* phenotype is epistatic to the *sli/robo* phenotype:** In *sli* null mutant embryos, the longitudinal tracts are collapsed at the midline in a fully penetrant manner (Kidd *et al.* 1999). This can be observed with Fas II and BP102 staining (Figure 3, A and E). When two mutants have opposing phenotypes, genetic epistasis studies have been most revealing in determining the hierarchy of gene activity and the relationship between the two genes in regulating a given process (Noordermeer *et al.* 1994; Siegfried *et al.* 1994; Bhat 1996; Bhat and Schedl 1997; Bhat *et al.* 2000). Since *net* and *sli* have opposing mutant phenotypes, we sought to determine the epistatic relationship between these two genes.

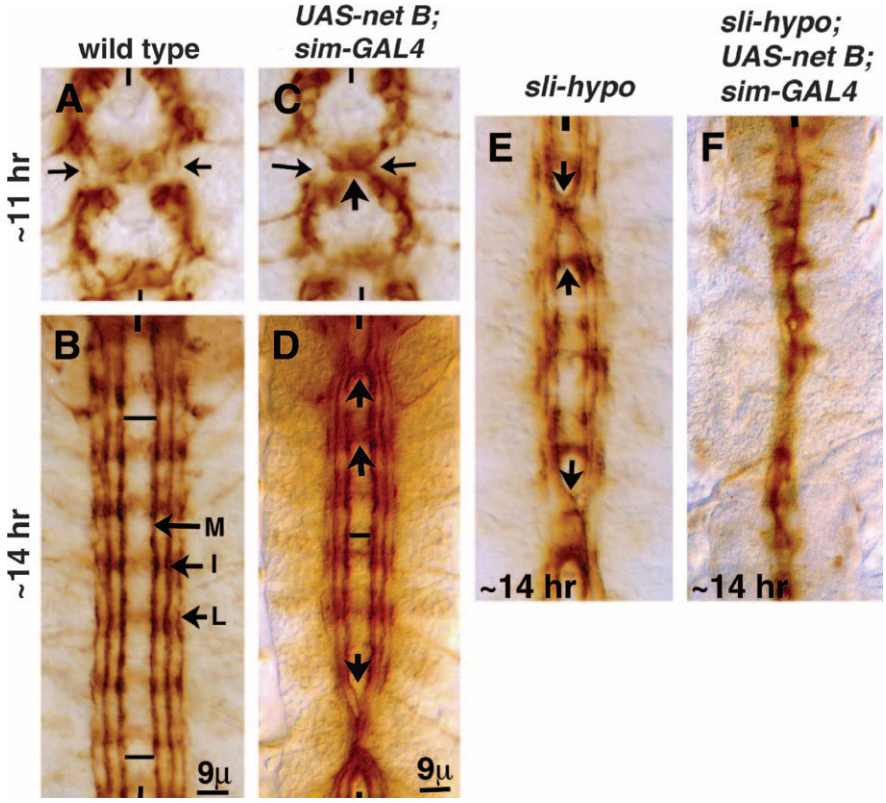

Figure 2.—Overexpression of Net at the midline attracts longitudinal tracts toward the midline. Anterior end is up; midline is marked by vertical lines. Embryos are stained for Fas II. (A) Wild-type ~11-hr-old embryo showing the projections from pCC neurons (arrows). (B) Wild-type ~14-hr-old embryo showing the three major Fas II tracts, medial (M), intermediate (I), and lateral (L). (C) An ~11-hr-old *UAS-net B/+; sim-GAL4/+* embryo. The projections from two pCC neurons on either side of the midline are attracted toward the midline (arrows). (D) An ~14-hr-old *UAS-net B/+; sim-GAL4/+* embryo. Arrows indicate the midline crossing of longitudinal tracts or their collapsing at the midline. (E) In an ~14-hr-old *sli* hypomorphic mutant embryo, mostly the medial tracts cross the midline (arrows). (F) An ~14-hr-old *UAS-net B/sim-GAL4; sli-hypo/sli-hypo* embryo.

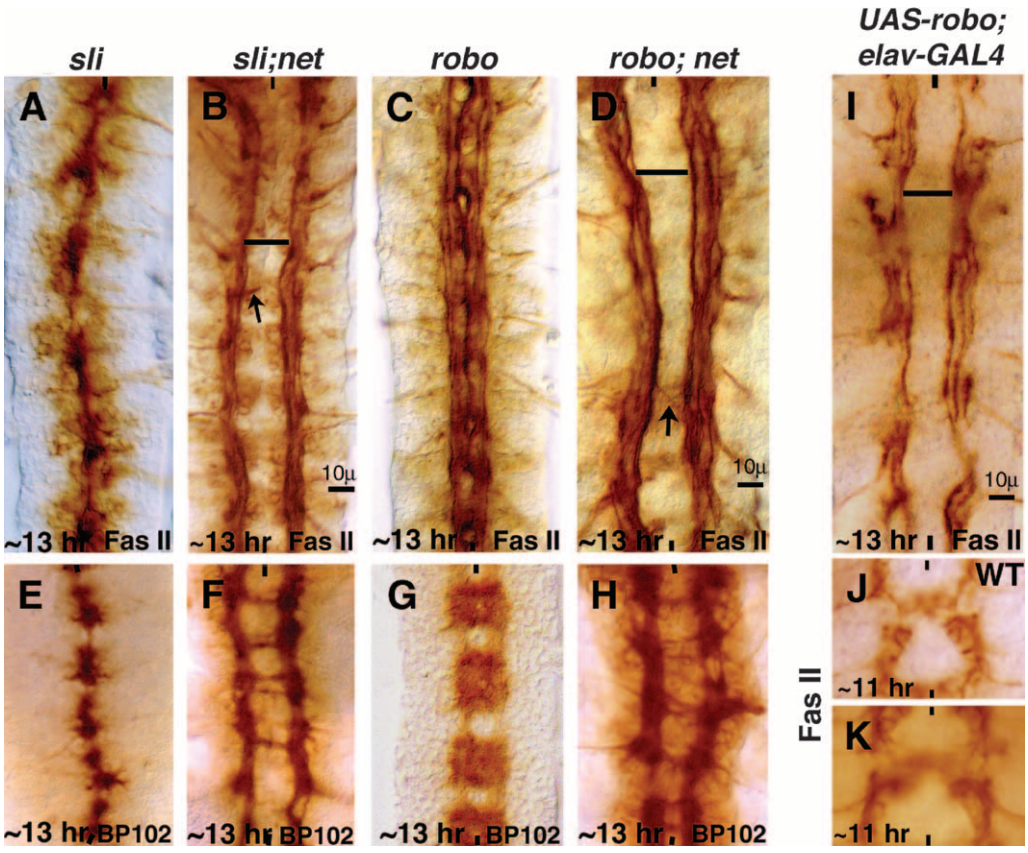

FIGURE 3.—*netrin* phenotype is epistatic to the *sli* and *robo* phenotypes. Anterior side is up; midline is marked by vertical lines. Embryos are ~14 hr old. Embryos in A–D are stained with Fas II. While in *sli* the longitudinal tracts are collapsed at the midline (A), in the *sli; net* double mutant (B), they are farther away from the midline as in a *net* single mutant. Similarly, while in *robo* (C) the medial tracts abnormally enter the midline, in the *robo; net* double mutant (D), they no longer enter the midline and the phenotype is the same as *net* single mutant. The distance between two medial tracts across the midline in wild type is ~10 μm and is indicated by a bar in B, D, and I. Arrows in B and D indicate midline crossing of longitudinal tracts. Embryos in E–H are stained with BP102. BP102 staining also shows that the *net* phenotype is epistatic to the *sli* or *robo* phenotypes. I–K are stained for Fas II. (I) An ~13-hr-old *UAS-robo/+; elav-GAL4/elav-GAL4* embryo. (J) An ~11-hr-old wild-type embryo. (K) An ~11-hr-old *UAS-robo/+; elav-GAL4/elav-GAL4* embryo (see text for details).

We generated *sli; net* double mutants and examined the positioning of longitudinal tracts in these embryos. As shown in Figure 3, while in *sli* the tracts are collapsed at the midline (Figure 3, A and E), in double mutants the positioning of tracts was as in *net* single mutants (Figure 3, B and F; see Table 1). This phenotype was fully penetrant as in *net* single mutant embryos ($n = -30$ embryos). This result indicates that the *net* phenotype is epistatic to the *sli* phenotype. One plausible interpretation of this result is that Net functions downstream of Sli and not in parallel (see also below). That is, in *sli* mutants Net at the midline exerts a strong attraction on growth cones, causing the tracts to collapse at the midline, whereas in the double mutant, since there is no Net-mediated attraction, growth cones no longer are attracted to the midline even when there is no Sli. In molecular terms, our above results suggest that Sli neutralizes Net-mediated attraction of growth cones toward the midline.

If Sli functions downstream of Net, we would have expected to observe a *sli* mutant phenotype in the *sli; net* double mutants. The third possibility is that Sli and Net function in a parallel pathway. In a parallel pathway, Net will attract lateral tract growth cones whereas Sli will repel the same growth cones independently of each other. Here, we expect to observe either an intermediate or a wild-type (or close to wild-type) lateral positioning of the longitudinal tracts in the double mutant. However, as shown in Figure 3B, the lateral positioning of longitudinal tracts is the same in the double mutant and the *net* single mutant. Therefore, while it is still possible that the two pathways are independent of each other, we believe that the neutralization model described above is probably the likely scenario (see below).

We next asked if the interaction between Robo and Sli is necessary to antagonize Net-mediated growth cone attraction from the midline. In *robo* mutants, the medial tracts inappropriately collapse at the midline (Figure 3, C and G). We generated *robo; net* double mutants and examined which of the phenotypes is epistatic in these embryos. As shown in Figure 3, D and H, these embryos also exhibited a *net* phenotype (see also Table 1), indicating that Robo and Sli interaction is necessary for Sli to oppose Net-mediated attraction. We also point out that, while longitudinal axons cross the midline in *net* single mutants in <1% of the hemi-segments ($n = 1100$;

data not shown), the frequency of longitudinal axons crossing the midline is slightly enhanced in both *sli; net* (3.5% of the hemi-segments, $n = 840$) and *robo; net* (3%, $n = 840$) double mutants (arrows in Figure 3, B and D). This suggests that the direct Sli-Robo-mediated repulsive signaling (Kidd *et al.* 1999) plays a contributory role in preventing longitudinal tracts from crossing the midline. We also examined *robo2; net* and *robo3; net* double-mutant embryos and in each of these cases, the *net* phenotype was epistatic (data not shown). This argues that both Robo2 and Robo3 also function via neutralizing the attraction by Net (see DISCUSSION).

Finally, since the neutralizing effect on Net-mediated attraction by Sli is Robo dependent, we reasoned that an excess of Robo (*robo* gain of function) in wild-type background should also lead to a loss of Net attraction. We expressed *robo* from a *UAS-robo* transgene in a pan-neural fashion using the elav-GAL4 driver and examined the positioning of longitudinal tracts in these embryos. As shown in Figure 3, I and K, in embryos overexpressing *robo* in a pan-neural fashion, the longitudinal tracts were positioned much more laterally and the phenotype resembled that of *net* mutant embryos ($n = 12$ embryos). This phenotype was the opposite of the one induced by the overexpression of *net* at the midline (see Figure 2, C and D).

Ectopic Slit fails to repel Robo-positive growth cones: Given the above possibility, we sought to further examine the role of direct repulsive interaction between Sli and Robo within the nerve cord and to determine if Sli can repel growth cones of Robo-positive pCC (an interneuron) and two motoneurons, aCC and RP2, when expressed ectopically in their projection paths. Specifically, we sought to reorient the longitudinal projection of pCC and ipsilateral and postero-lateral projections of RP2 and aCC [loss of Sli or Robo activity causes pCC to project toward the midline in a fully penetrant manner (Kidd *et al.* 1999) whereas the abnormal projection of aCC and RP2 toward the midline is observed in ~15% of the hemi-segments (see Wolf and Chiba 2000)] by ectopically expressing Sli using the UAS-GAL4 system in front of their projection paths. The drivers *patched (ptc)-GAL4* (Figure 3B) and *armadillo (arm)-GAL4* (Figure 4c) were used to ectopically express Sli from a *UAS-sli* transgene (see supplementary information at http://www.genetics.org/supplemental/ for the expression pattern of *ptc* and *arm*). We also introduced a *UAS-GAL4* transgene to maintain *sli* expression at high levels continually prior to and during axonogenesis following the initial induction by an autoregulatory loop (Hassan *et al.* 2000). In these experiments, the growth cones of aCC and RP2 ignored the ectopic Sli stripe and projected normally (Figure 4, e, h, f, and i; b and c show levels of ectopic Sli at about the same time that RP2 begins to grow an axon; $n = 336$ hemi-segments). The levels of ectopic Sli in these stripes are as high as in the midline (Figure 4, b and c) (the *ptc* and *arm* drivers are active as early as ~4 hr of development, see Bhat 1996; see supplementary results at http://www.genetics.org/supplemental/). Similarly, the growth cone of pCC also projected normally (Figure 4, k and j; $n = 336$ hemi-segments) as in wild type, contrary to the expectation that the ectopic Sli stripe would repel its growth cone and we would observe breaks in the medial tracts with anti-Fas II staining (*cf.* Schindelholz *et al.* 2001). These results indicate that Sli-Robo interaction either does not always lead to a repulsive output or requires additional inputs that are not available outside of the midline. On the other hand, these results also support the possibility that Sli-Robo interaction regulates positioning of axon tracts mostly by neutralizing Net-mediated attraction.

It was possible that ectopic expression of *sli* in stripes causes a downregulation of Robo; thus, these growth cones cannot respond to Sli. However, expression of Robo was unaffected in these embryos (data not shown). Moreover, a *robo*-like phenotype was also not observed, as one would have expected if Robo were downregulated. Since these UAS-*sli* transgenes rescue *sli* mutant phenotype (data not shown; see Kidd *et al.* 1999), it is unlikely that ectopic Sli produced by these transgenes is nonfunctional.

The blocking of Net-mediated attraction of growth cones by Sli-Robo signaling occurs downstream of Frazzled: The blocking of Net-mediated attraction of growth cones by Sli-Robo can occur in several ways. Previous results have shown that, in stage 22 Xenopus spinal neurons, binding of Sli to Robo leads to an interaction between the cytoplasmic domains of Robo and Fra, which in turn silences the Fra-Net-mediated attraction (Stein and Tessier-Lavigne 2001; see Figure 5A). If this scenario is correct, availability of an excess of Fra should overcome the inhibitory interaction between Robo and Fra and the neutralizing effect on Net-mediated attraction and therefore should lead to a collapsing of the longitudinal tracts at the midline. Therefore, initially we overexpressed *fra* in wild-type background in a pan-neural manner. This did not result in any axon guidance phenotype (Figure 5D; $n = 30$ embryos). Next, we overexpressed *fra* in the hypomorphic $sli^{E\text{-}158}$ mutant background (in this allele a *P*-element is inserted in the 5′ region of the gene; thus, only the expression of the gene is affected but not the protein itself). This should lead to an enhancement of the collapsing of longitudinal axon tracts at the midline of *sli* hypomorphic mutants. However, no such enhancement was observed (Figure 5F; $n = 30$ embryos). Furthermore, increasing the copy numbers of *fra* using a duplication chromosome also had no effect ($n = 30$ embryos). These are negative results; however, since the *fra* transgene rescues the *fra* mutant phenotype when expressed using the *elav-GAL4* driver (each in single copy; see also Keleman and Dickson 2001), this transgene does produce functional Fra protein and the negative results are likely to be meaningful.

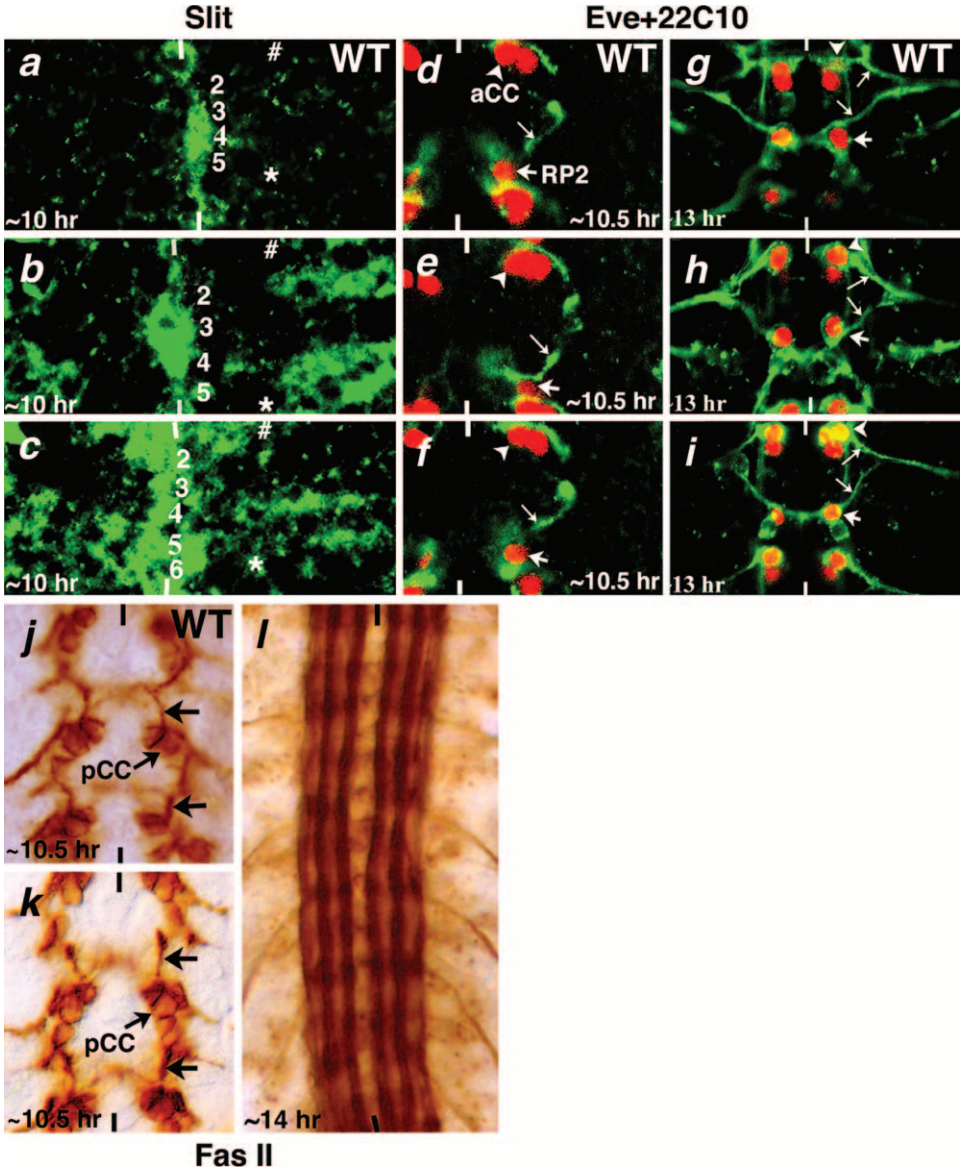

FIGURE 4.—Striped ectopic expression of sli in front of the projection paths of RP2, aCC, and pCC does not alter their projection pattern. Embryos in a–c are stained for Sli. In d–i, embryos are double stained for Eve (red, nuclear) and 22C10 (green, membrane). Embryos in j–l are stained with anti-Fas II. Anterior end is up; midline is indicated by the vertical lines. Numbers along the midline in a–c indicate rows of neuroblasts. In a–c, the positions of aCC and RP2 neurons during the time of axonogenesis are marked by a number sign and an asterisk, respectively. Large arrows mark the RP2, small arrows indicate axon projection from RP2 and aCC, and arrowheads indicate aCC. (a) Wild-type embryo. Sli is only in the midline cells. (b) *UAS-sli; UAS-GAL4; ptc-GAL4* embryo. Sli is also present in rows of cells above RP2 and above pCC. (c) *UAS-sli; UAS-GAL4; arm-GAL4* embryo. Sli is present in rows above RP2 and below aCC. (d) Wild-type embryo. Projections from aCC and pCC neurons have not yet fasciculated with each other. (e and f) *UAS-sli; UAS-GAL4; ptc-GAL4* and *UAS-sli; UAS-GAL4; arm-GAL4* embryos. In e and f, the axon projections of RP2 and aCC are not yet fasciculated but they are similar to wild type and not repulsed by the ectopic Sli stripes. (g) Wild-type embryo. Both aCC and RP2 projections have fasciculated together. (h and i) *UAS-sli; UAS-GAL4; ptc-GAL4* and *UAS-sli; UAS-GAL4; arm-GAL4* embryos. Both aCC and RP2 have normal projection patterns and are not repulsed by the ectopic Sli stripes. Note that we could not simultaneously examine these embryos with Sli and 22C10 antibodies since both these primary antibodies are mouse antibodies. (j) An ~10.5-hr-old wild-type embryo showing pCC and its projection. (k) An ~10.5-hr-old *UAS-sli; UAS-GAL4; ptc-GAL4* embryo. The projection from pCC is not affected. (l) An ~14-hr-old *UAS-sli; UAS-GAL4; ptc-GAL4* embryo. No breaks in the longitudinal tracts were observed.

A previous study found that Sli binds to Net *in vitro* (BROSE *et al.* 1999). It is therefore possible that binding of Robo to Sli leads to a physical interaction between Sli and Net and silencing of Net-Fra interaction (Figure 5B). However, this possibility, *in vivo*, seems unlikely for two reasons. First, overexpression of *sli* at the midline fails to cause any phenotype (see KIDD *et al.* 1999; our unpublished results). In the above scenario, it is expected that too much of Sli would neutralize the attraction and the tracts will be positioned farther away from the midline. Second, the pan-neural overexpression of *fra* in wild-type and *sli* hypomorphic mutant backgrounds did not result in the attraction of axon tracts toward the midline (Figure 5, D and F; $n = 12$ embryos). Under the above scenario, there is more of Net available in the *sli* mutant and, thus, overexpression of *fra* should have caused an enhancement of the midline collapsing defect in *sli* mutant embryos. These results, therefore, argue that Robo signaling interferes indirectly with the Net-Fra signaling. That is, an interaction between Sli with Robo leads to an interference of Net-Fra-mediated attraction downstream of Fra (Figure 5C). Therefore, overexpression of either Fra or Sli need not affect the positioning of axon tracts.

The effect of Robo^{extra}-Fra^{intra} chimeric protein on longitudinal tracts is dependent on the presence of Net activity: In a previous reciprocal domain-swapping experiment, BASHAW and GOODMAN (1999) found that

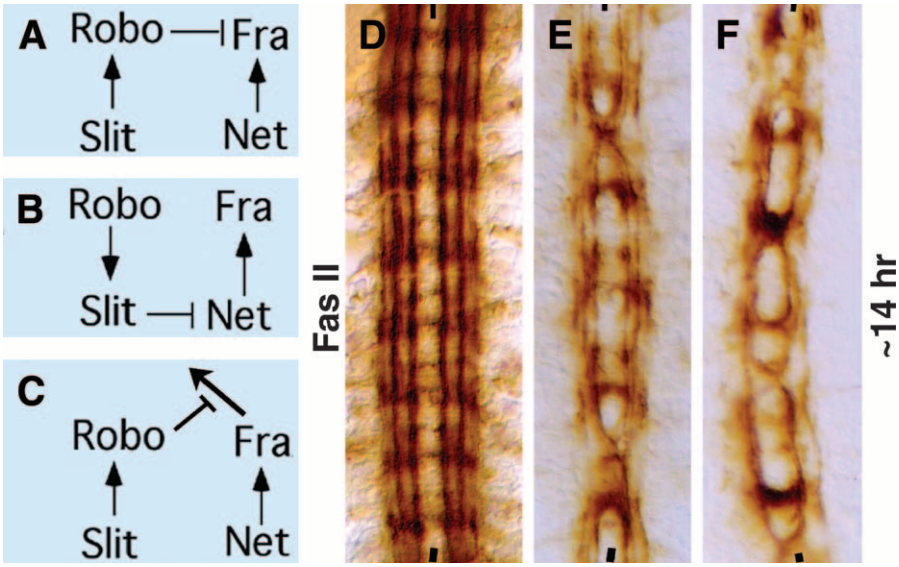

FIGURE 5.—Interaction between Robo, Sli, Net, and Fra during the positioning of longitudinal and commissural tracts. There are at least three alternative possibilities for the interaction of these proteins. In the first scenario (A), Robo prevents Fra-Net interaction in a Sli-dependent way. In the second (B), Sli prevents Net-Fra interaction in a Robo-dependent way. In the third (C), Robo neutralizes the Fra-mediated attractant signaling at the downstream level. These possibilities are examined in D and F (see text for details). Embryos in D–F are stained for Fas II. Anterior end is up; midline is marked by vertical lines. (D) An ~14-hr-old *UAS-fra; elav-GAL4* embryo. Pan-neural expression of Fra has no effect. (E) An ~14-hr-old *sli* hypomorphic embryo. (F) An ~14-hr-old *sli-hypo/sli-hypo; UAS-fra/elav-GAL4* embryo. There was no worsening of the phenotype in *sli* hypomorphic mutant CNS with pan-neural overexpression of Fra.

expression of a chimeric protein between the extracellular domain of Fra and the intracellular domain of Robo (Fra^{extra}-$Robo^{intra}$) caused a repulsion of commissural tracts. On the other hand, expression of a chimeric protein between the extracellular domain of Robo and the intracellular domain of Fra ($Robo^{extra}$-Fra^{intra}) caused midline crossing of longitudinal tracts and recrossing of commissural tracts. We sought to determine if the midline crossing of longitudinal tracts in $Robo^{extra}$-Fra^{intra} embryos is dependent on the presence of an intact Net activity. In the direct Sli and Robo repulsive signaling scenario, the clear prediction is that the *sli*-like phenotype of $Robo^{extra}$-Fra^{intra} would be epistatic to the *net* phenotype. In our model, we would expect the *net* phenotype to be epistatic. We generated embryos of the genotype UAS-$Robo^{extra}$-Fra^{intra}/elav-GAL4; UAS-$Robo^{extra}$-Fra^{intra}/+; *net*/Y and examined their longitudinal axon guidance phenotype by anti-Fas II staining. These embryos had the *net* phenotype (Figure 6B; $n = 12$ embryos) indicating that the *net* phenotype is epistatic to the $Robo^{extra}$-Fra^{intra} phenotype (Figure 6A).

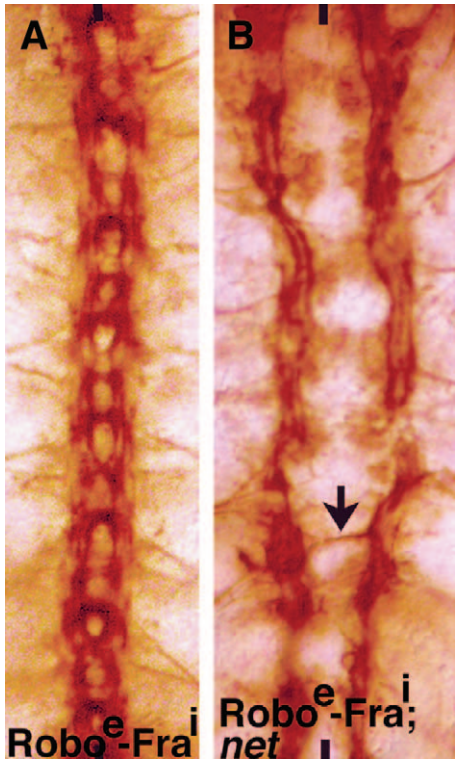

FIGURE 6.—Growth cone attraction mediated by the chimeric protein between Robo and Fra is net dependent. Embryos are stained with anti-Fas II. Anterior end is up; midline is marked by vertical lines. (A) UAS-$Robo^{extra}$-Fra^{intra}/elav-GAL4; UAS-$Robo^{extra}$-Fra^{intra}/+ embryo. (B) UAS-$Robo^{extra}$-Fra^{intra}/elav-GAL4; UAS-$Robo^{extra}$-Fra^{intra}/+; *net*/Y embryo. See also DISCUSSION, Figure 7, and its legend for more details.

DISCUSSION

Sli-Robo signaling neutralizes attraction of growth cones of the longitudinal tracts by Net-Fra and specifies their lateral position: The precise lateral positioning of the longitudinal axon tracts along the VNC in the Drosophila embryo is a very important paradigm to study how growth cones navigate in a diverse environment and find their synaptic targets. Several previous studies have concluded that a direct combinatorial repulsive interaction between Sli secreted from the midline glial cells and the three different Robo proteins on growth cones specifies the lateral positioning of longitudinal tracts along the midline (BASHAW and GOODMAN 1999; KIDD *et al.* 1999; RAJAGOPALAN *et al.* 2000; SIMPSON

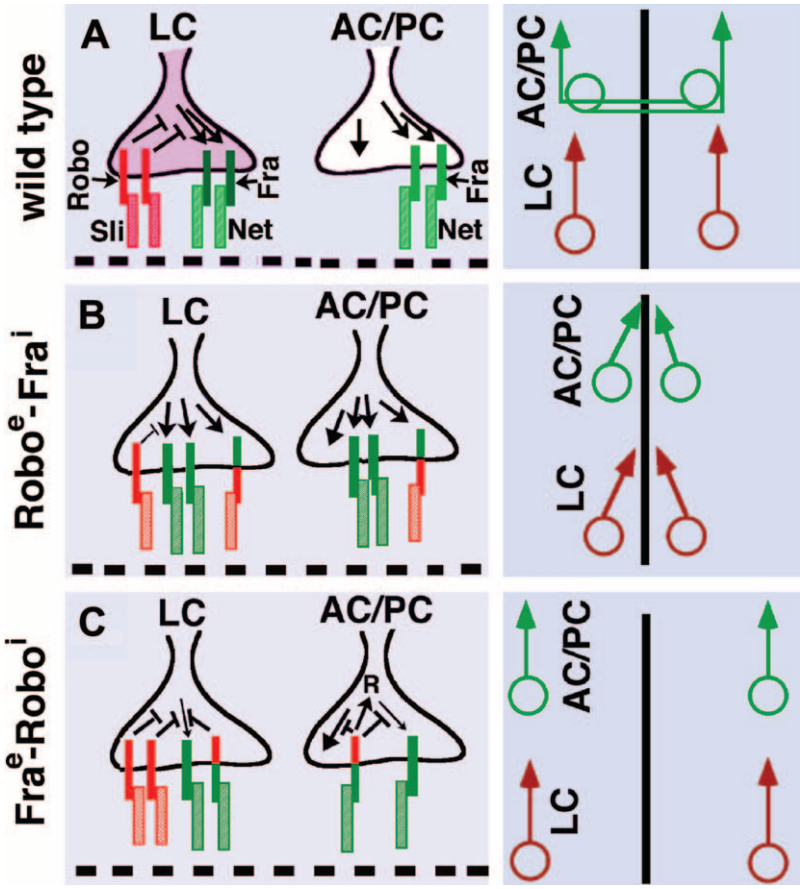

FIGURE 7.—Interaction between Sli-Robo and net-Fra signaling pathways. (A–C) Schematics of growth cone of longitudinal connective (LC), anterior commissure (AC), and posterior commissure (PC) along with localization of Robo (solid red bar), Sli (hatched red bar), Net (light green bar), and Fra (dark green bar). Midline is marked by a dashed line. (A) In LC, Robo-Sli interaction blocks Net-Fra pathway downstream of Fra. In AC and PC where growth cones project toward the midline, the growth cones lack Robo; thus, Net-Fra pathway is able to mediate attraction. Additional attractant cues must also exist since *net* or *fra* mutant embryos do not display a complete commissureless phenotype. (B) In LC, with the pan-neural overexpression of $Robo^{extra}$-Fra^{intra}, there is a reduction in the neutralizing effect of Net-Fra attraction by Sli-Robo due to the dominant negative effect of the chimeric protein and an increase in the attraction mediated by the Fra^{intra} causing a *sli*-like phenotype. In AC and PC, there is an enhancement of the attraction due to the chimeric Robo-Fra signaling. (C) In LC, with the pan-neural overexpression of Fra^{extra}-$Robo^{intra}$, the Net-Fra-mediated attraction is negatively affected in two ways: a dominant negative effect and an increase in the neutralizing effect of the attraction. This results in the axon tracts positioned farther away. In AC/PC, not only does the ectopic expression of the chimeric protein neutralize the Net-Fra attraction, but also the $Robo^{intra}$ neutralizes the additional attractant cues since the commissureless phenotype is very strong. The commissureless phenotype may also be due to a significant contribution from a Sli-Robo-mediated direct repulsive signaling (see text).

et al. 2000). However, the results described in this article bring out another possibility that the Sli-Robo signaling neutralizes the attractant cue mediated by the Net-Fra signaling to specify the lateral positioning of longitudinal pathways (Figure 7A). Thus, the inappropriate entry of longitudinal growth cones and their collapsing at the midline in *sli* mutants could be due to the loss of silencing of attraction by Net-Fra signaling. Since Robo is downregulated in commissural tracts (TEAR *et al.* 1996) and therefore there is no silencing of the Net-Fra attractant cue, Net-Fra signaling is able to mediate attraction of commissural axons toward the midline (Figure 7A).

What is the evidence to support the above model? First, loss of function for *net* or *fra* causes a more lateral shift in the position of the longitudinal tract (Figure 1), indicating that Net-Fra exerts an attractant force on these tracts. Second, overexpression of *net* at the midline causes an inappropriate projection of longitudinal growth cones toward the midline and the collapsing of the lateral tracts at the midline (a similar overexpression of *sli* at the midline has no consequence, see also KIDD *et al.* 1999). Moreover, such an expression of *net* in a *sli* hypomorphic mutant background enhances the midline collapsing of lateral tracts (Figure 2). These indicate that enhanced levels of Net can overcome silencing by Sli-Robo signaling. Third, the *net* phenotype was epistatic to the *sli* phenotype and the longitudinal tracts were more laterally placed as in *net* mutant embryos in *net; sli* or *net; robo* or *fra; sli* and *fra; robo* double-mutant combinations (Figure 3). When there is no attraction mediated by Net-Fra signaling, loss of *sli* or *robo* does not cause longitudinal growth cones to inappropriately enter the midline. Therefore, Sli-Robo signaling is necessary to prevent these tracts from entering the midline only when there is attraction by Net-Fra. These epistatic results also suggest that this is not a parallel pathway, but it is a linear pathway where Net is downstream of Sli. Fourth, ectopic expression of Sli in front of the growth cones of Robo-expressing neurons fails to prevent these growth cones from projecting normally toward their targets (Figure 4). For example, pCC, which sends out one of the pioneering axons for the medial longitudinal tract, normally extends its growth cone despite having ectopic Sli in front of it. Similarly, projections from an RP2 or aCC (motoneurons) also fail to respond to ectopic Sli. All three neurons express Robo and are affected in *sli* mutants. Moreover, the transgene used in these ectopic expression experiments can rescue the loss-of-function *sli* mutant phenotype (KIDD *et al.* 1999). Therefore, the inability of these growth cones to respond to ectopic Sli argues against a direct repulsive scenario and supports the model where Sli-Robo signal-

ing specifies the lateral position of longitudinal tracts by silencing Net-Fra-mediated attraction.

Finally, expression of a chimeric protein between the extracellular domain of Robo and the intracellular domain of Fra ($Robo^{extra}$-Fra^{intra}) causes midline crossing of longitudinal tracts (Bashaw and Goodman 1999). If the direct repulsion scenario is correct, this chimeric protein should convert the direct repulsion into a direct attraction (as was proposed, see Bashaw and Goodman 1999). Therefore the $Robo^{extra}$-Fra^{intra} phenotype should be epistatic to the *net* phenotype. But expression of this chimeric protein in a *net* mutant background suppressed the inappropriate midline crossing of longitudinal tracts and these embryos had the *net* phenotype (Figure 6).

How can we explain the midline crossing of longitudinal tracts and recrossing of commissural tracts observed with the expression of a $Robo^{extra}$-Fra^{intra}? In the longitudinal connectives (LCs), the chimeric protein contributes to the attraction mediated by endogenous Net-Fra signaling and, as a result, the silencing effect of endogenous Sli-Robo signaling is overcome (Figure 7B). In the absence of endogenous Net activity in $Robo^{extra}$-Fra^{intra}; *net* embryos, the attraction mediated by the $Robo^{extra}$-Fra^{intra} chimeric protein is neutralized by the endogenous Sli-Robo signaling. Furthermore, the two chimeric proteins have strong dominant negative effects (Bashaw and Goodman 1999). Thus, while it has been claimed previously that this chimeric protein actively attracts axons to the midline using the attractive activity of the Fra intracellular domain, it is also possible that the effect of the fusion protein reflects titration of Slit by the Robo extracellular domain, without the Fra moiety doing anything active at all. If that is the case, the fusion protein is acting as a dominant negative and behaving as a *sli* hypomorphic mutation and the epistatic result between *net* and this chimeric line does not add any new information.

On the other hand, Bashaw and Goodman (1999) argue that the dominant negative effects of the chimera are minimal. If this is true, at the minimum, the dominant negative effect will contribute to the inappropriate midline crossing phenotype observed in $Robo^{extra}$-Fra^{intra} embryos. This possibility is further supported by the finding that overexpression of Fra does not lead to any guidance phenotypes. Furthermore, the same dominant negative effect will also reduce the ability of endogenous Sli signaling to neutralize the attraction mediated by the $Robo^{extra}$-Fra^{intra} protein in $Robo^{extra}$-Fra^{intra}; *net* embryos. (The above *sli*-like phenotype in $Robo^{extra}$-Fra^{intra} embryos further argues against a direct Sli-Net binding scenario since such a binding would create a loss of attraction and thus a *net* or *fra*-like phenotype.) In anterior commissure (AC) and posterior commissure (PC) where growth cones project toward the midline (Figure 7B), the growth cones lack Robo; thus, the Net-Fra pathway is able to mediate attraction. Additional attractant cues must also exist since *net* or *fra* mutant embryos do not display a complete commissureless phenotype.

Expression of a chimeric protein between the extracellular domain of Fra and the intracellular domain of Robo (Fra^{extra}-$Robo^{intra}$) causes a more lateral positioning of longitudinal tracts (Figure 7C; see Bashaw and Goodman 1999). This is expected since the chimeric protein in this case will contribute to a more efficient silencing of the Net-Fra attraction (Figure 7C). Again, since this chimeric protein also has strong dominant negative effect (Bashaw and Goodman 1999), the attraction by the endogenous Net-Fra signaling is likely to be weaker in embryos expressing pan-neural Fra^{extra}-$Robo^{intra}$ compared to wild type. In the longitudinal tracts (LC), this reduced attraction is compounded by a silencing of the attraction mediated by the intracellular domain of Robo, resulting in tracts positioned farther away from the midline.

In AC or PC, the pan-neural expression of Fra^{extra}-$Robo^{intra}$ leads to a commissureless phenotype (Bashaw and Goodman 1999). Since the phenotype in this case appears to be similar to the *commissureless* (*comm*) mutant phenotype [where Robo is upregulated in the commissural tracts (Seeger *et al.* 1993; Tear *et al.* 1996)], Fra^{extra}-$Robo^{intra}$ must block not only the attraction mediated by Net-Fra but also any additional attractant signaling on commissural tracts mediated by some unknown players. It is also highly likely that the Robo-Sli direct repulsive signaling, unlike in the positioning of longitudinal tracts, significantly contributes to the guidance of commissural tracts (denoted by an R in Figure 7B) and, thus, the Fra^{extra}-$Robo^{intra}$ mimics a *comm* mutant phenotype.

Robo combinatorial code and Sli-Net interaction: How does the Robo combinatorial code fit in with the Sli-Net results described in this article? Specifically, overexpression of Robo2 and Robo3, for example, drives the medial tracts more lateral (Rajagopalan *et al.* 2000; Simpson *et al.* 2000); how do we account for this result if the Sli signaling positions the lateral tracts via silencing of Net signaling? We believe that these results are consistent with the scenario in which the Sli-Robo interaction blocks events downstream of Fra. That is, a combination of signaling by Robo receptors interferes with the downstream events mediated by Fra. Driving Robo2 in medial tracts, for example, would interfere with the Fra signaling in such a way that the medial tract now adopts a more lateral tract trajectory. This is also consistent with the finding that the *net* phenotype is epistatic to the *robo2* or *robo3* phenotypes (data not shown). Therefore, both Robo2 and Robo3 must function via blocking the attraction by Net.

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